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#21

FACSIMILE COVER SHEET

TO:	Examiner Anne Kubelik Art Unit 1638
FACSIMILE NUMBER:	703-872-9306
FROM:	Alan J. Howarth, Ph.D.
DATE:	March 26, 2003
RE:	U.S. Patent Application No. 09/576,623
YOUR REFERENCE NO.:	
OUR FILE NO.:	T4088.cont
NUMBER OF PAGES:	22 (INCLUDING COVER PAGE)

FAX RECEIVED
MAR 27 2003
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I confirm that the inventor, Dr. John Carman, and I will meet with you at 10:00 am on Friday, March 28, 2003, for an in-office interview. We want to focus on the enablement issue. Attached is a print-out of a Power Point presentation that we will bring with us. We do not necessarily intend to present the PowerPoint presentation. We are bringing it to illustrate certain points that we may discuss.

If you do not receive the complete document, please call **(801) 255-5335** as soon as possible. Our fax number is **(801) 255-5338.**

Presentation Outline

- Prior art vs new discoveries (1990s)
  - Anatomy, evolution and inheritance
  - Producing and stabilizing apomictic plants
- Patent-pending methods enabled by new discoveries
- Enablement
  - Producing apomictic plants (1st pending patent) by:
    - Locating and characterizing embryologically-divergent lines
    - Breeding lines such that embryological stages overlap
    - Characterizing lines for apomixis
  - Stabilizing apomixis (2nd pending patent) by conferring structural heterozygosity or by preventing segregation
  - Controlling apomixis (2nd pending patent) by controlling sexual reproduction

"Prior art" refers to the apomixis embryology knowledge base available to the inventor at the time the discoveries and inventions were made.

"New discoveries" refers to the apomixis knowledge base that was discovered and developed by the inventor, which enabled the methods of the 1st and 2nd pending patents.

The enablement methods of both pending patents are straightforward logical extensions of the "new discoveries." They are not anticipated by the prior art.

By following the methods described in the 1st pending patent, we have produced apomictic plants (plants that produce apomictic embryo sacs at low to high frequencies) from sexual plants (those that do not produce apomictic embryo sacs or do so at very low frequencies, <1% of ovules) in three of three taxa (100%) attempted to date (*Antennaria*, *Tripsacum* and *Sorghum*). No undue experimentation was required.

By following the methods of the 2nd patent, we have genetically stabilized a genetically unstable synthetic apomict.

Apomictic plants have neither been created by man predictively nor stabilized by man predictively prior to these inventions.

By following the methods of the 1st and 2nd pending patents, those skilled in the art can produce apomictic plants from sexual plants and stabilize them without undue experimentation.

Prior Art vs New Discoveries (1990s)

Anatomy, Evolution and Inheritance of Apomixis

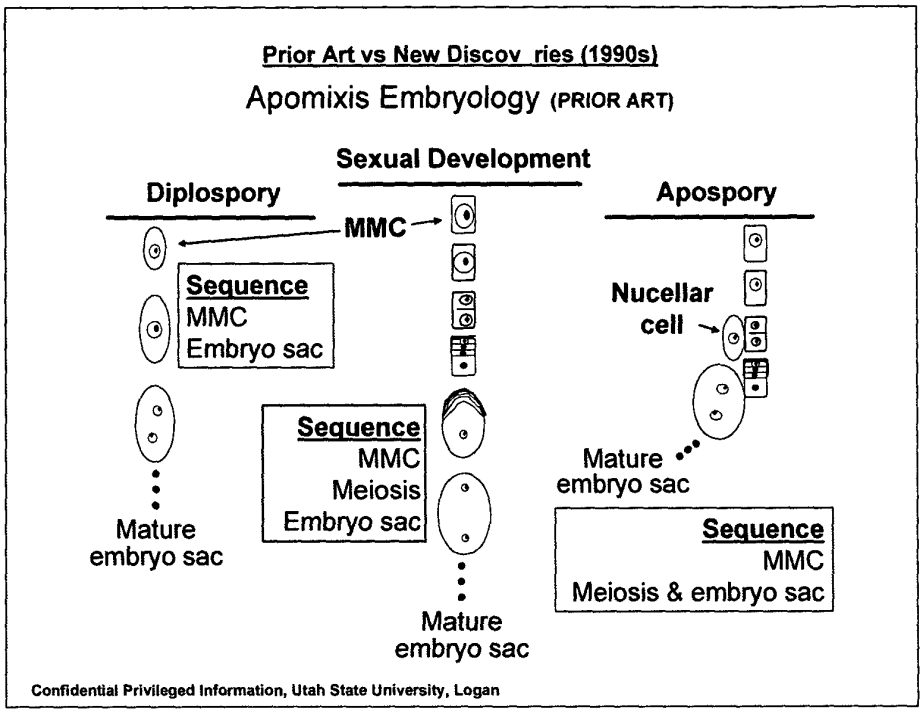
1. Anatomical phenomena that characterize gametophytic apomixis (PRIOR ART)
   - Two forms of apomixis with several different types in each
     - diplospory
     - apospory
   - Absence of callose around diplosporous MMC
     - published in the late 1980s and 1990s
     - diplospory (apomictic) and tetraspory (sexual) are identical in this trait (discovered in the inventor's lab)
     - an important discovery leading to the "new discoveries"
   - Sub-cellular (ultrastructural) anatomical studies
     - published in the 1980s and 1990s
     - apomictic embryo sac formation often is identical to sexual embryo sac formation except that the nuclei are unreduced

Notes and References

Johri et al. 1992. Comparative Embryology of Angiosperms, Vol 1: **see 1-4**, general information; **29-41**, monospory and polyspory; **84-94,** adventitious embryony and gametophytic apomixis; **Asker and Jerling, 1992**, Apomixis in Plants, **see 49-80**, Mechanisms of Apomixis. *These papers review apomixis embryology.*

Carman et al 1991. Comparative histology of cell walls during meiotic and apomeiotic megasporogenesis in two australasian *Elymus* L. species. Crop Sci. 31:1527-1532; **Peel et al 1997.** Megasporocyte callose in apomictic buffelgrass, Kentucky bluegrass, *Pennisetum squamulatum* Fresen, *Tripsacum* L. and weeping lovegrass. Crop Sci. 37:724-732. *These papers document absence of callose in diplosporous MMC of various natural apomicts.*

Naumova and Willemse, 1995, Ultrastructural characterization of apospory in *Panicum maximum,* Sex Plant Reprod 8: 197-204; **Naumova et al 1999**, Apomixis in plants: structural and functional aspects of diplospory in *Poa nemoralis* and *P. palustris,* Protoplasma 208:186-195. *These papers document ultrastructural findings regarding apomictic embryo sac formation.*



Notes and References

The apomixis development sequences (diplospory and apospory) diagrammed here were elucidated in the first 15 to 20 years of the 20th Century (for reviews, see **Johri et al. 1992** ibid, **Asker and Jerling, 1992** ibid).

For a recent review of the inheritance of these apomictic sequences, see **Sherwood 2001** (Genetic analysis of apomixis, in Savidan et al ed, The Flowering of Apomixis: From Mechanisms to Genetic Engineering, D.F.: CIMMYT, IRD, EC DG VI, FAIR).

Though simple inheritance for apospory and diplospory have been demonstrated in several taxa, the specifics concerning the evolution of these anomalies, and the specific nature of the gene(s) involved, have remained highly speculative (see next slide)

The most widely accepted concept in the prior art concerning the nature of the genes that cause apomixis is that they are mutations that specifically cause apomixis (see next slide).

Prior Art vs New Discoveries (1990s)

Anatomy, Evolution and Inheritance of Apomixis

2. Evolution of apomixis

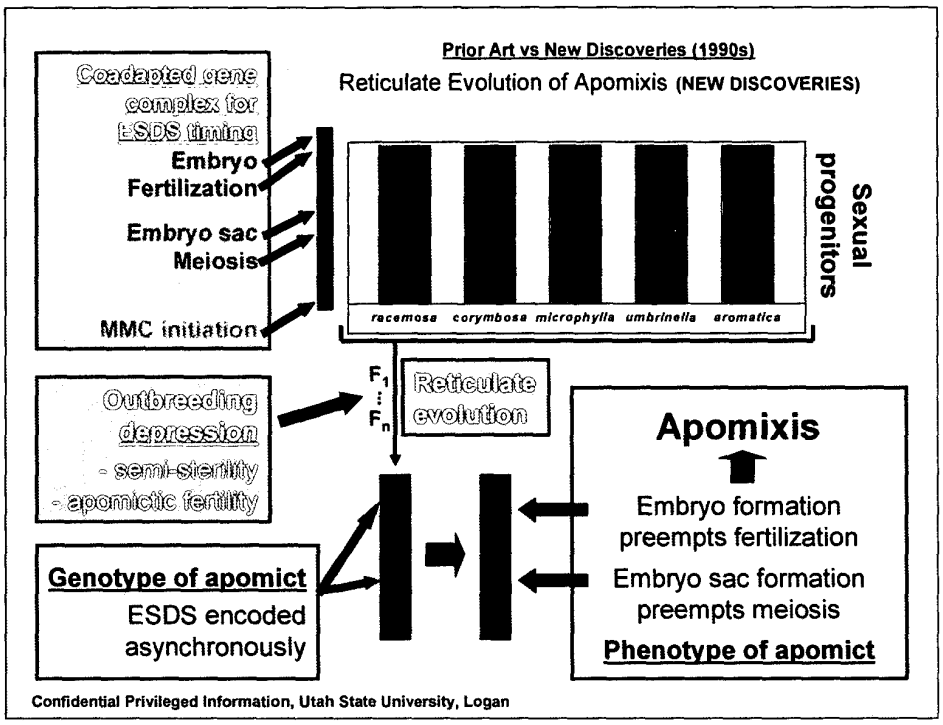
- Conventional models (PRIOR ART)
  - wide hybridization causes apomixis (1912 through 1940)
  - specific apomixis genes, derived by mutation, cause apomixis (1940 to present)
- Models enabling pending patents (1994 – 1999) (NEW DISCOVERIES)
  - due to adaptive radiation, ecotypes often differ in onset times and durations of megasporogenesis, embryo sac formation and embryony
  - secondary contact hybridization often combine alleles of ecotypes that differ in onset times and durations of various embryological stages
  - in hybrids or their early generation segregates, unique combinations of alleles may cause apomixis (embryo sac formation to preempt meiosis and embryony to preempt fertilization) (See Fig 1, pg 52-53, Carman 1997)
  - multiple-ecotype-derived allelic combinations responsible for fledgling apomicts are occasionally stabilized by structural heterozygosity

Notes and References

Gustafsson Å. 1947. Apomixis in higher plants. III. Biotype and species formation. Lunds Universitets Årsskrift 43: 181-370. *This paper discusses early models for the evolution of apomixis,* ***see 295-303****.*

Mogie M. 1992. The evolution of asexual reproduction in plants. London: Chapman and Hall. *This book discusses early models for the origins of apomixis,* ***see 139-144****; conventional mutation-based model,* ***see 144-196****.*

Carman JG. 1997. Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. Biol J Linnean Soc 61: 51-94. *This paper presents, for the first time in the open literature, the model of the first pending patent* ***(Fig 1, 52-53)****, which depicts asynchronous expression of genes, from the MMC stage to the early embryo development stage. The asynchronous expression in hybrids or their early generation derivatives is caused by the presence of divergent alleles from genomes of divergent parental ecotypes.*



Reticulate evolution = developmental changes resulting from repeated recombination of genes between previously isolated taxa **(PRIOR ART DEFINITION)**

ESDS = embryo sac development sequence, i.e. onset times and durations of megasporangia formation, megasporogenesis (female meiosis), megagametogenesis (female gametophyte or embryo sac formation), fertilization, and early embryony relative to nongametophytic tissues of the ovule or ovary **(the acronym "ESDS" is unique to the inventor's technology)**

Outbreeding depression = outbreeding-induced disruptions of coadapted gene complexes resulting in reduced fertility or performance (see **Montalvo & Ellstrand 2001**, for a current review of this phenomenon)

Figure Caption: Apomixis origins according to the Reticulate-evolution Structural-heterozygosity (RS) model. The diagram depicts divergent ESDS phenotypes observed among sexual progenitors of apomictic *Antennaria rosea.* ESDS phenotypes are encoded by coadapted gene complexes that evolve by intense habitat speciation (adaptive radiation). Secondary contact hybridization followed by reticulate evolution randomly recombines (or shuffles) divergent alleles of the divergent gene complexes. Three segregant classes may arise: 1) those with unresolved ESDS asynchronies, which cause sexual sterility or semisterility (outbreeding depression), 2) those with resolved ESDS asynchronies, which are sexually fertile, and 3) those with unique ESDS asynchronies that cause apomixis (asexual fertility). In the latter, embryo sac formation preempts meiosis and embryony preempts fertilization. *This model enables the de novo production of apomictic plants.*

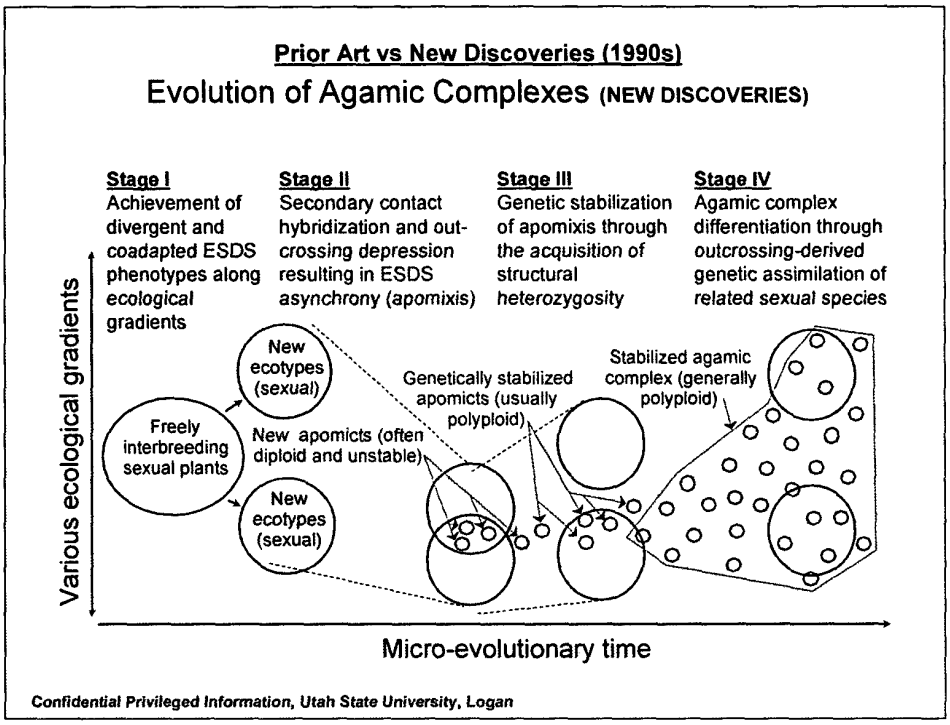


Figure Caption: Reticulate-evolution structural-heterozygosity (RS) model for the evolution of agamic complexes. Stages I and II involve reticulate evolution, i.e. evolutionary changes resulting from repeated recombination of genes between previously-separated ecotypes. Stage III involves genetic stabilization of loci responsible for apomixis through karyotypic heterozygosity mechanisms (see next slide). Stage IV involves genetic assimilation of related sexual species (agamic complex diversification), the capacity for which is enhanced by the karyotypically-stabilized complex loci that confer apomixis.

ESDS = embryo sac development sequence, i.e. onset times and durations of megasporangia formation, megasporogenesis, megagametogenesis, fertilization, and early embryony relative to nongametophytic tissues of the ovule or ovary.

Genetic stability of apomixis = a property of natural or man-made facultative apomicts (plants capable of sexual and apomictic reproduction) that causes sexually-derived progeny (occasionally produced facultatively) to be as apomictic as the mother plant though otherwise genetically recombined. Without genetic stability, sexually derived progeny tend to revert to complete or nearly complete sexuality. This occurs because of genetic segregation at various heterozygous loci that contain unique alleles responsible for apomixis (**the term "genetic stability of apomixis" is unique to the inventor's technology).**

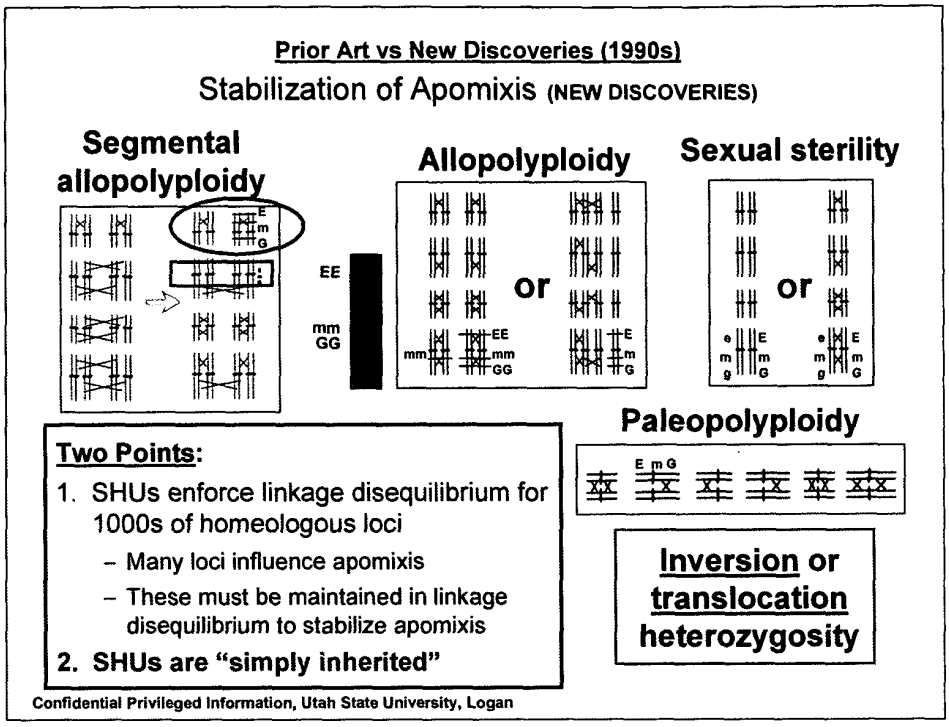
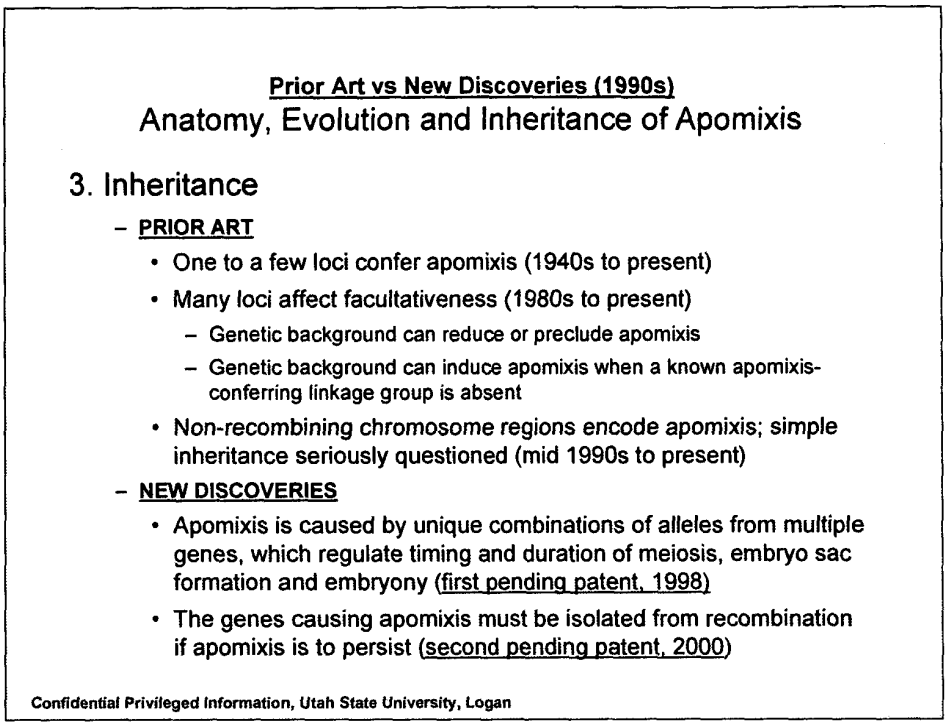


Figure Caption: Karyotypic heterozygosity stabilizes the linkage disequilibrium responsible for apomixis. For genetic stability to exist, linkage disequilibrium for one or more groups of linked loci must be maintained, not only through generations of progeny produced asexually (via apomixis) but through generations of progeny facultatively produced through normal sexual processes. The mechanisms that accomplish this stabilization effectively link tens to thousands of genes together into complex loci (supergene clusters) in which recombination is suppressed.

Asexual seed formation is a form of genetic stabilization of apomicts. This is probably one of several reasons why most apomicts found in nature are high frequency to near-obligate apomicts. Bispory and tetraspory are sexual anomalies that do not have the luxury of asexual reproduction. Plants exhibiting these anomalies are diploidized polyploids **(Carman 1997)**. The polygenic heterozygosity required for bispory and tetraspory to be "genetically stabilized" is maintained by the karyotypic (or structural) heterozygosity conferred by chromosome base number expansion (paleopolyploidy).

Karyotypic heterozygosity is probably a major underappreciated mechanism of evolution of novel forms and functions in plants and animals, many of which could not persist without genetic stabilization of polygenic heterozygosity.



Notes and References

Mogie, 1992, The evolution of asexual reproduction in plants (**see 144-196**); **Sherwood et al, 1994**, Inheritance of apospory in buffelgrass, Crop Sci 34:1490-1494; **Leblanc et al, 1995**, Detection of the apomictic mode of reproduction in maize-*Tripsacum* hybrids using maize RFLP markers, Theor Appl Genet 90: 1198-1203. *These papers teach single gene inheritance.*

Carman JG, 2000, The evolution of gametophytic apomixis, In Batygina (ed) Embryology of Flowering Plants, Vol. 3, The Systems of Reproduction, Russian Acad Sci, St. Petersburg. *This paper reviews 1980s to mid 1990s papers that document wide variation in facultativeness,* ***see 230-236.***

Grimanelli et al, 1998, Mapping diplosporous apomixis in tetraploid *Tripsacum*: one gene or several genes, Heredity 80:33-39; **Ozias-Akins et al, 1998**, Tight clustering and hemizygosity of apomixis-linked molecular markers in *Pennisetum squamulatum* implies genetic control of apospory by a divergent locus that may have no allelic form in sexual genotypes, Proc Natl Acad Sci 95: 5127-5132. *These papers suggest that the "single gene" is a simply-inherited supergene that may contain multiple "apomixis genes."*

1st and 2nd Pending Patent Applications, 1998, 2000. The first pending patent provides strong evidence that the genes that cause apomixis are normal genes with multiple divergent alleles that are found in divergent ecotypes. The second pending patent provides strong evidence that the unique combinations of alleles from linked and unlinked loci that cause apomixis must be protected from recombination. The two pending patents provide predictable methods for synthesizing apomictic plants and genetically stabilizing them.

Prior Art vs New Discoveries (1990s)

Producing and Stabilizing Apomictic Plants

1. Strategies for producing apomictic plants **(PRIOR ART)**
   - Transfer "apomixis gene(s)" to a sexual plant from an apomictic relative by breeding (introgression) or genetic engineering
   - Mutate a gene(s) of a sexual plant and select apomictic mutants
   - Identify and clone normal genes that control embryological development and "engineer" them to cause apomixis
2. Strategies for stabilizing apomictic plants **(NO PRIOR ART EXISTS)**
   - Stabilization of the unique combinations of alleles that cause apomixis, from linked and unlinked loci, is a unique concept. We are not aware of literature that contemplates the need for such manipulations
   - Frequency apomictic seed formation drops to near zero in plants in which the unique combinations of alleles, which cause apomixis, are not faithfully inherited from the facultatively-apomictic mother plant
   - Stabilization involves strategies that prevent allelic segregation at the linked and unlinked loci involved in conferring high frequency apomixis

Ramula et al 1999, Apomixis for crop improvement, Protoplasma 208: 196-205 (**see Abstract and Conclusions**); **Jefferson & Bicknell 1996**, The potential impacts of apomixis: a molecular genetics approach, in *The Impact of Plant Molecular Genetics*, Birkhauser, Boston (**see bottom of 88 – 89, 94, 98 "Misleading..."**); **Kultunow et al 1995**, Apomixis: molecular strategies for the generation of genetically identical seeds without fertilization, Plant Physiol 108: 1345-1352 (**see 1347 "Genetic Basis"**). *These papers discuss conventional strategies for attempting to produce apomictic plants (introgression of apomixis genes, mutation, gene engineering). Conventional approaches rely on the hypothesis that specific apomixis genes exist or can be produced by mutation or engineering.*

Jefferson & Bicknell 1996 (see 98 "Misleading..."); Mogie, 1992, The evolution of asexual reproduction in plants (**see bottom of 144 and 145**); *These authors come the closest of any we are aware of in contemplating a need for genetic stabilization as defined in the second pending patent. Jefferson and Bicknell reason that if apomixis is caused by multiple mutations, any one of which would be lethal if segregated out by itself, then mutations in nature will tend to be linked such that apomixis-conferring linkets will mimic a simply-inherited single-gene locus ("Misleading Single-locus Behavior"). Mogie reasons that apomixis could not involve many individually-lethal mutations or it could not have evolved. The second pending patent differs from the prior art in that (a) wild-type genes from multiple linked and unlinked loci (not lethal mutations at a few loci) must be shielded from recombination if high frequency apomixis is to be stabilized and (b) recombination causes reversion primarily to sexuality, not lethality (apomixis is not caused by mutated genes).*

Patent-pending Methods Enabled by New Discoveries

1. Producing apomictic plants (1st pending patent)
   - Mimic evolution of apomixis in nature by bringing genomes together from plant lines that are divergent in timing of meiosis, embryo sac formation and embryony (degree of divergence is critical and is specified in the pending patent)
   - Modify the genetic background by breeding lines or hybrids so that embryo sac formation preempts meiosis and embryony preempts fertilization, i.e. so that apomixis can occur
2. Stabilizing & controlling apomixis (2nd pending patent)
   - Stabilize apomixis by:
     - conferring structural heterozygosity mechanisms that isolate the major genes that cause apomixis and the modifiers that influence facultativeness from recombination and subsequent segregation; or
     - preventing segregation altogether by eliminating sexual reproduction
   - Control apomixis by allowing or preventing sexual reproduction

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Examples 5 & 7 of the 1st pending patent provide specific guidance for producing apomictic plants from sexual plants. Ecotypes of the same or closely related species are chosen that "represent latitudinal and other ecological extremes." Choosing divergent ecotypes facilitates finding lines that possess sufficient variation in start times and durations of critical embryological stages. The lines are embryologically characterized (described in example 5), and lines divergent in timing are identified. Example 7 states: "lines are selected such that the initiation of embryo sac formation (degenerating megaspore stage) in one set of lines (usually the long-day-adapted lines) occurs at about the same time as female meiotic prophase through metaphase is occurring in the other set of lines relative to the development of the nongametophytic tissues of the ovule and ovary."

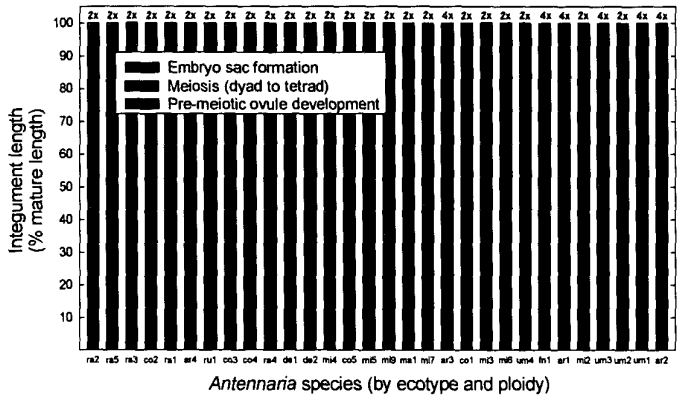
Flowering response used primarily to assist in locating lines that differ in embryological timing.

By following these steps, plants that produce apomictic embryo sacs have been produced, in our program, from plants that do not produce apomictic embryo sacs. We have succeeded, without undue experimentation, in three of three taxa tried to date (*Antennaria*, *Sorghum* and *Tripsacum*). The predictive production of plants that undergo apomictic embryo sac formation from plants that produce only sexual embryo sacs has never before been accomplished.

Example 2 of the 2nd pending patent provides an example of producing a genetically stabilized triploid apomict (obligately apomictic due to triploidy) from a facultative tetraploid apomict.

Enablement: 1st Pending Patent

Step 1: Finding Embryologically-divergent Lines



A. umbrinella at the **dyad stage** of megasporogenesis (integuments are about 50% mature)

A. racemosa at the **dyad stage** of megasporogenesis (integuments are about 20% mature)

Figure Caption: ESDS phenotypes of 30 ecotypes from nine sexual *Antennaria* species. Phenotypes are based on start of meiosis, defined as attainment of the dyad stage (bottom of red line), and duration of meiosis, defined as duration from the dyad stage to the 1-nucleate embryo sac stage (length of red line). Data was taken from about 6000 ovules. Species are: ra, *racemosa*; co, *corymbosa*; ru, *rosulata*; de, *densifolia*; mi, *microphylla*; ma, *marginata*; ar, *aromatica*; fn, *friesiana alaskana*; um, *umbrinella*. *A. racemosa*, *A. corymbosa*, *A. microphylla*, *A. aromatica* and *A. umbrinella* are believed to be the sexual progenitors of apomictic *A. rosea* (Bayer 1996).

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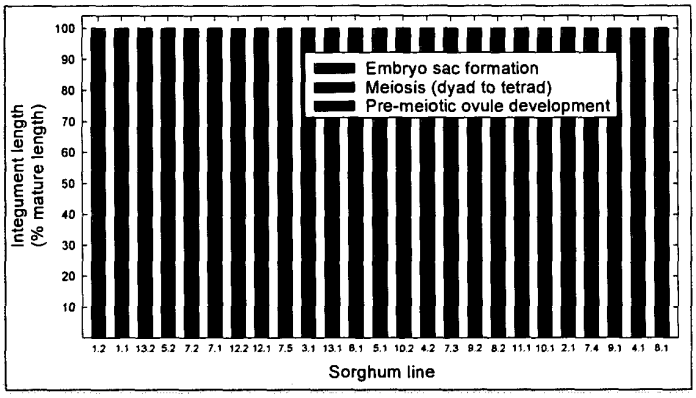
The 1st pending patent was enabled by the discovery that schedules of embryological development in ovules often very substantially among ecotypes of the same or closely related species **(NEW DISCOVERIES)**. These data are as yet unpublished in that additional patentable methods for modifying ESDS timing are being verified. Once ESDS variability was discovered, methods were devised to efficiently identify two or more lines that collectively possess the allelic divergence required to induce apomixis. The method for identifying such lines was included in Example 5 and states: "*...the following data are obtained for each ovule analyzed: meiotic or embryo sac development stage, pistil length and width, inner and outer integument lengths, and meiocyte or embryo sac length and width... Developmental stage data are graphed against (a) pistil and integument lengths and widths (raw data) and (b) the lengths and widths of these structures represented as percentages of their mature lengths and widths (measured at stigma exertion). The likeness of ecotypes with respect to female developmental schedules is tested by analysis of variance, and diagrams ... are produced ...*" We have performed such analyses in three sets of related taxa, *Sorghum, Tripsacum* and *Antennaria*, and have initiated such analyses in *Arabidopsis*. This slide plus the following three slides document the variation in female developmental scheduling (relative to nongametophytic tissues of the ovule, e.g. integument growth stages) found to date.

Enablem nt: 1st Pending Patent

Step 1: Finding Embryologically-divergent Lines



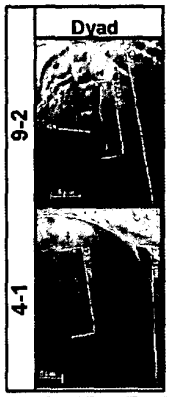


Figure caption: ESDS phenotypes (a - h) for 25 *Sorghum* lines screened to date. Phenotypes are based on start of meiosis, defined as attainment of the dyad stage (bottom of red line), and duration of meiosis, defined as duration from the dyad stage to the 1-nucleate embryo sac stage (length of red line). Data was taken from about 5000 ovules. Note that embryo sac formation in phenotype 'a' begins before the dyad stage is reached in phenotypes b, e and h. 1.1, 1.2, Aispuri and Aispuri converted (C); 2.1, Kafir; 3.1, IS 3922; 4.1, 4.2, Karad Local (C), Karad Local; 5.1, Early Kalo; 5.2, Westland; 6.1, Colby; 7.1, 1111; 7.2, C120; 7.3, O 756; 7.4, Zhuronskiya; 7.5, IS 11010; 8.1, IS 12693; 8.2, R-319; 9.1, VIR-5049; 9.2, Agira; 10.1, Lydenburg Red; 10.2, IS 13856; 11.1, Chaondera; 12.1, IS 36854; 12.2, Gharib; 13.1, Ba Ye Qi; 13.2, Nian Gaoliang.

Figure caption: The dyad stage occurs early in 4-1 but late in 9-2 relative to integument maturity.

The ESDS variation necessary to induce apomictic embryo sac formation in *Sorghum* was discovered after screening only 25 accessions of over 30,000 *Sorghum* accessions maintained in international sorghum germplasm banks.

Note that embryo sac formation in phenotype 'a' begins at about the same time as or before the dyad stage is reached in phenotypes b, e and h (a criterion for producing apomictic plants from sexual plants as specified in Example 7 of the 1st pending patent).

Enablement: 1st Pending Patent

Step 1: Finding Embryologically-divergent Lines

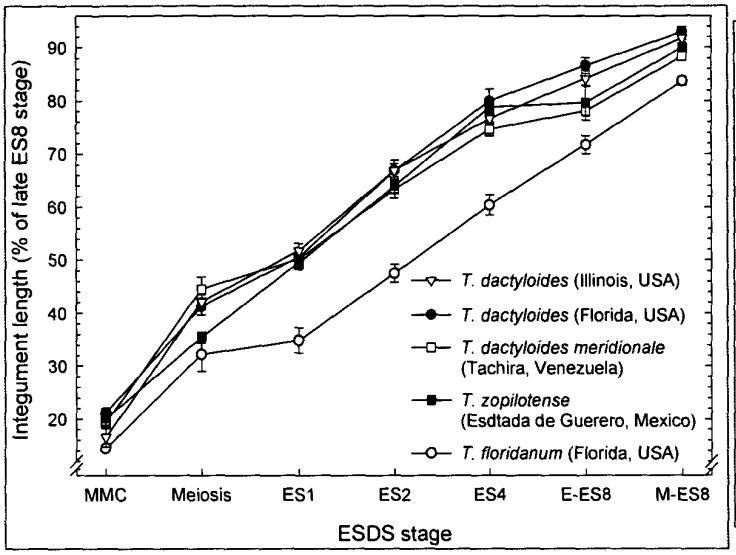


Figure Caption: ESDS phenotypes of five ecotypes from four sexual diploid *Tripsacum* species. Phenotypes are based on MMC stage, start of meiosis (attainment of the dyad stage), embryo sac 1 (ES1) stage, ES2, ES4, early ES8 and mid ES8 (bars are standard errors). *Tripsacum dactyloides* and *T. floridanum* are thought to be sexual progenitors of apomictic tetraploid *T. dactyloides* (Tantravahi 1968).

Note that embryo sac formation (attainment of the 1-nucleate embryo sac stage) in *T. floridanum* occurs before the dyad stage of megasporogenesis in *T. dactyloides* (a criterion for producing apomictic plants from sexual plants as specified in Example 7 of the 1st pending patent).

Breeding *T. floridanum* with *T. dactyloides* combines, in the hybrids, alleles for early embryo sac formation with alleles for late meiosis. Plants that produce low to moderate frequencies of diplosporously-apomictic embryo sacs (MMCs produce genetically unreduced embryo sacs instead of going through meiosis) have been selected from the F_1 generation of this cross (presented below). We expect that higher frequency apomictic embryo sac formation will occur among the F_2 to F_4 segregates, i.e. through the methods of the patent, conventional breeding and selection, we intend to eliminate alleles for early meiosis and alleles for late embryo sac formation. The resulting plants should then undergo much higher frequencies of apomictic embryo sac formation.

Enablement: 1st Pending Patent

Step 1: Finding Embryologically-divergent Lines

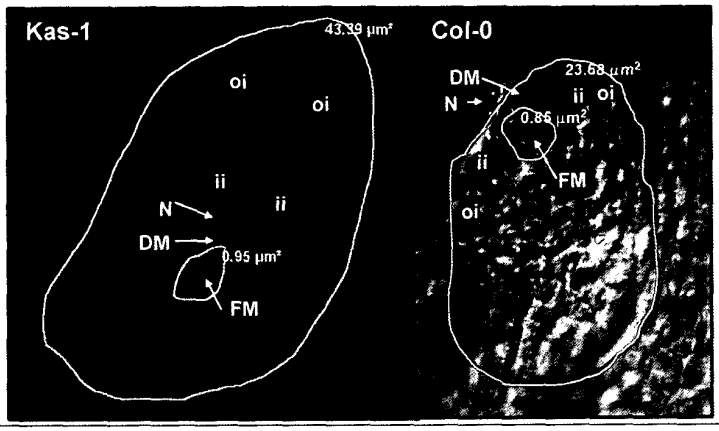


Figure caption. Differential interference microscopy images showing ESDS divergence at the functional megaspore stage (onset of embryo sac formation) between *Arabidopsis* lines Kas-1 and Col-0. Col-0 *consistently initiates embryo sac formation earlier in ovule development than Kas-1. In Kas-1, the integuments have* completely grown around and well above the nucellus and have reached approximately 80% of their mature length. In Col-0, the integuments are approximately 15% of their mature length (compare with variation observed in *Antennaria). Ovule measurements (large circles) include the integuments down to the top of the funiculus.* FM=functional megaspore, DM=degenerating megaspores, N=nucellus, ii=inner integument, oi=outer integument.

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The methods of the 1st pending patent were recently applied to *Arabidopsis*, which is a sexual diploid. However, apomixis does exist in *Arabis*, a close relative of *Arabidopsis*. At present, we have documented in *Arabidopsis* the embryological (ESDS) variation necessary to induce apomixis (see figure above), and the mapping and cloning of ESDS genes responsible for apomixis is underway.

Enablement: 1st Pending Patent

Step 2: Breeding and Selecting for Apomixis

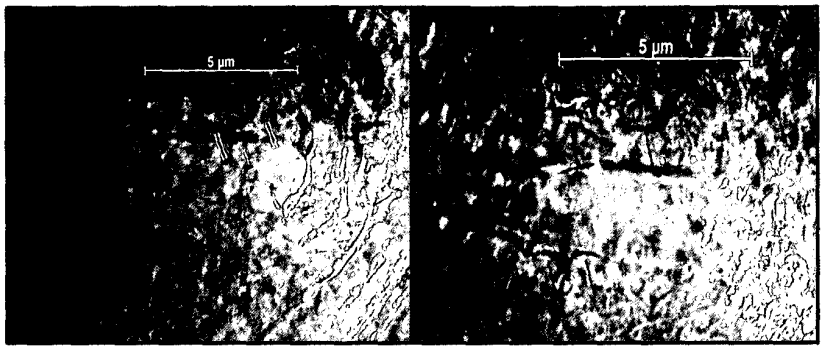

Figure caption: Two focal planes of a 1-nucleate aposporous embryo sac in *Sorghum bicolor* hybrid 9.1 x 1.2 (see slide 12). Left image: micropylar region of the sexual tetrad and aposporous embryo sac. Double white arrows point to vacuoles in the aposporous embryo sac; white arrows point to the nucleus; black arrow points to the degenerating micropylar member of the linear tetrad. Right image: chalazal region of the tetrad and aposporous embryo sac. Double black arrows point to the degenerating functional megaspore of the sexual linear tetrad. Single black arrows point to degenerating spores (two upper-most single black arrows) and nucellar cells surrounding the growing aposporous embryo sac.

As discussed above, the 1st pending patent was enabled by the discovery that schedules of embryological development vary among ecotypes. Once this was discovered, methods were devised to identify lines that collectively possess the allelic divergence required to induce apomixis (described in Example 5 and demonstrated above). Example 7 describes the types of lines that must be included in a breeding program to produce apomictic plants from sexual plants. Example 7 includes the following instructions: "*lines are selected such that the initiation of embryo sac formation (degenerating megaspore stage) in one set of lines (usually the long-day-adapted lines) occurs at about the same time as female meiotic prophase through metaphase* [approximately the dyad stage] *is occurring in the other set of lines relative to the development of the nongametophytic tissues of the ovule and ovary.*" We have made such selections in three sets of related taxa.

Starting with diploid sexual *Tripsacum*, we have bred lines (diploid and polyploid) that produce apomictic embryo sacs at low (10 - 20% of pistils) to high (nearly 100% of pistils) frequencies. From sexual *Sorghum* and *Antennaria*, we have bred lines (diploid and polyploid) that produce apomictic embryo sacs at low to moderate frequencies (10 - 30%). Apomictic embryo sacs rarely form (< 1%) in the parent lines. We are currently conducting progeny tests to determine the frequency of apomictically-derived progeny from our *Sorghum* lines. Apomictic embryo sac formation in our man-made *Sorghum* and *Tripsacum* apomicts is morphologically identical to that observed in fully functional apomictic embryo sacs in nature. Hence, we believe apomictic seed set will occur.

This slide plus the following three slides document production of plants that produce apomictic embryo sacs at moderate frequencies (10% to 90% of ovules) from sexual plants that seldom produce apomictic embryo sacs (<1%).

Enablement: 1st Pending Patent

Step 2: Breeding and Selecting for Apomixis

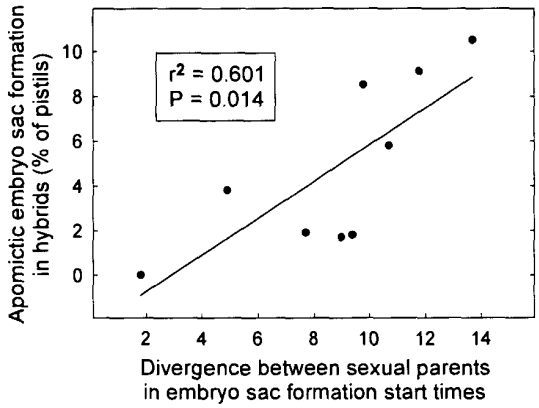

Figure caption. Regression between parent line divergence in embryo sac initiation and frequency aposporous embryo sac formation in hybrids. Five to six sibs were analyzed per hybrid combination. The sib with the highest frequency aposporous embryo sac formation per hybrid combination (>30 appropriately-staged pistils per sib) was used in the analysis. The pairs of parents for each hybrid combination were strongly divergent in meiosis duration.

We quantified variation among *Sorghum* lines in ESDS start times and durations as described in Example 5 of the first patent (see Slide 12). We then made hybrids between sexual parents that differed in embryo sac formation start times (Slide 12, top of red line) and meiosis duration (length of red line) such that embryo sac formation start times in one parent were overlapping meiosis in the other parent (as specified in Example 7 of the 1st pending patent). We then analyzed the hybrids for aposporous embryo sac formation (see slide 15), which occurred at various frequencies among the progeny. Degree of divergence in meiosis start times and duration explained 36% of the variability ($r^2 = 0.36$, positively correlated) among 28 hybrids produced and analyzed to date (different parent-line combinations) in percentage pistils (per hybrid) in which aposporous embryo sacs were forming (highly significant, regression P = 0.004). In a subset consisting of nine of the 28 hybrids, those whose parents were in the upper 50th percentile for divergence in meiosis duration, variation in embryo sac formation start times explained 60% of the variability (see figure above).

Enablement: 1st Pending Patent

Step 2: Breeding and Selecting for Apomixis

- **Diplospory** requires embryo sac formation to precede meiosis
- **Apospory** requires embryo sac formation to strongly overlap meiosis

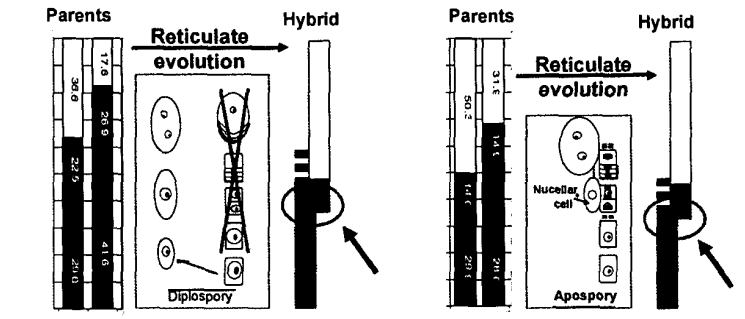

Figure caption: ESDS asynchronies required for apospory differ from those required for diplospory. In *Sorghum*, percentage diplosporous embryo sac formation in hybrids is positively correlated with divergence between parents in meiosis start times (bottom of red line) and use of female semisterile parents ($r2 = 0.12$, $P = 0.067$). As shown in the previous slide, percentage aposporous embryo sac formation is positively correlated with embryo sac start times and divergence in meiosis duration.

As discussed in the 1st pending patent, apomixis is caused by two reversals in the normal sequence of ovule development: 1) a meiosis vs embryo sac formation reversal and 2) a fertilization vs embryogenesis reversal. By selecting and breeding *Sorghum* plants divergent in timing of meiosis and embryo sac formation (according to the methods of Examples 5 and 7), we have produced *Sorghum* hybrids that undergo either apospory or diplospory. We followed the methods of the patent by observing the extend of divergence among parents in embryological timing, and we have now noted that the type of apomixis expressed, apospory or diplospory, involves more subtle gene interactions. For example, *Sorghum* hybrids that tend to express diplosporous embryo sac formation arise when sexual embryo sac formation in one parent precedes meiosis in the other. In contrast, aposporous embryo sac formation tends to occur in *Sorghum* hybrids when sexual embryo sac formation in one parent strongly overlaps meiosis in the other parent (see diagram above).

Enablement: 1st Pending Patent

Step 2: Breeding and Selecting for Apomixis

- Percentage apomictic embryo sac formation in ***Sorghum*** hybrids (compare with slides 12, 15-17)
  - 1.1/9.2 (10% aposporous)
  - 2.1/6.1 (7.2% total: 5.5% aposporous, 1.7% diplosporous)
  - 4.1/3.1 (8.5% aposporous)
  - 4.1/6.1 (8.3% total: 5.8% aposporous, 2.6% diplosporous)
  - 5.1/4.1 (9.1% aposporous)
  - 5.2/9.2 (13.5% diplosporous)
  - 9.1/1.2 (13.8% aposporous)
  - 9.2/10.1 (19.4% diplosporous)

The methods of the 1st pending patent were applied to *Sorghum* to breed plants that produce apomictic embryo sacs (at least some of which should be capable of producing asexual seed) from plants that generally produce only sexual embryo sacs (>99% sexual embryo sac formation): 1) plants originating from divergent habitats were ESDS-characterized and screened for apomictic embryo sac formation, 2) sexual plants divergent in ESDS timing were selected and bred, and 3) hybrids that initiate apomictic embryo sac formation in at least 7% of their ovules were identified through embryological screening.

Enabl ment: 1st Pending Patent

Step 2: Breeding and Selecting for Apomixis

- Percentage apomictic embryo sac formation in ***Antennaria*** hybrids (see slide 11)
  - *A. aromatica/pulchella* (30% diplosporous)
  - *A. umbrinella/umbrinella* (15% diplosporous)
  - *A. corymbosa/racemosa* (5% diplosporous)
  - *A. marginata/racemosa* (5% aposporous initials, occasional autonomous endosperm)

Figure caption. Sexual and diplosporous embryo sac formation in an *A. corymbosa* x *A. racemosa* hybrid. a,b: sexual MMC and tetrad, respectively; c,d: diplosporous MMC with vacuoles and 2-nucleate embryo sac without remnants, respectively.

The methods of the 1st pending patent were applied to *Antennaria* to breed plants that produce apomictic embryo sacs (at least some of which should be capable of producing asexual seed) from plants that generally produce only sexual embryo sacs (>99% sexual embryo sac formation): 1) plants originating from divergent habitats were ESDS-characterized and screened for apomictic embryo sac formation, 2) sexual plants divergent in ESDS timing were selected and bred, and 3) hybrids that initiate apomictic embryo sac formation in at least 5% of their ovules were identified through embryological screening.

Enablement: 1st Pending Patent

Step 2: Breeding and Selecting for Apomixis

- Percentage apomictic embryo sac formation in ***Tripsacum*** hybrids (see slide 13)
  - *T. floridanum/dactyloides* (Florida): 2% aposporous, 10% diplosporous
  - *T. floridanum/dactyloides* (Illinois): 13% aposporous, 8% diplosporous
  - *T. floridanum/zopilotense*: 3% aposporous, 5% diplosporous
  - *T. floridanum/dactyloides meridionale*: 1% aposporous
  - *laxum/pilosum* amphiploid//*bravum*: 5% diplosporous
  - *laxum/pilosum* amphiploid//*laxum*: 87% diplosporous

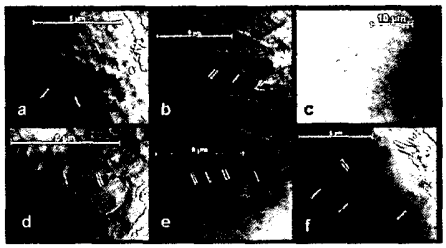

Figure caption. Aposporous and diplosporous embryo sac formation in synthetic *Tripsacum* apomicts produced from sexual diploids. a, aposporous initials (white arrows) and a degenerating tetrad (black arrows); b,d, diplosporous MMC, i.e. 1-nucleate diplosporous embryo sac (single arrows point to the nucleus, double arrows point to a prominent vacuole, which do not occur in sexual MMC); c, parthenogenic embryo (large circular structure) and undivided central cell (to the left); e, f, two and 4-nucleate diplosporous embryo sacs (single arrows point to nuclei, double arrows point to prominent vacuoles)

The methods of the 1st pending patent were applied to *Tripsacum* to breed plants that produce apomictic embryo sacs (at least some of which should be capable of producing asexual seed) from plants that generally produce only sexual embryo sacs (>99% sexual embryo sac formation): 1) plants originating from divergent habitats were ESDS-characterized and screened for apomictic embryo sac formation, 2) sexual plants divergent in ESDS timing were selected and bred, and 3) hybrids that initiate apomictic embryo sac formation in at least 1% of their ovules were identified through embryological screening.

Enablem nt: 2st P nding Patent

Example: Conferring Genetic Stability by Triploidy

- Obligate apomixis
  - optimization of competence components
  - optimization of signal components
- Obliteration of female meiosis
  - odd ploidy (see figure)
  - female meiotic mutants
  - transformation

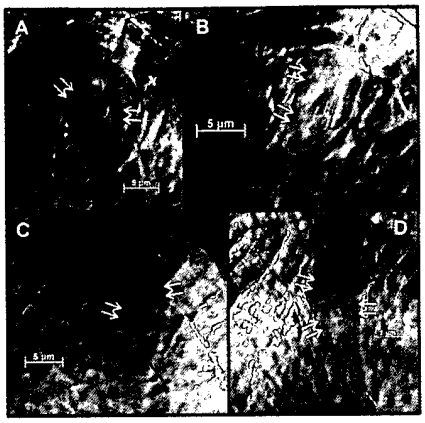

Figure Caption. Diplosporous embryo sac formation in an obligately-apomictic synthetic *Tripsacum* triploid BC1. A, MMC vacuolation (1-nuc diplosporous embryo sac, DES1); B, apomeiotic anaphase I in a DES1; C, DES2; D, DES4 with two micropylar nuclei in focus (chalazal boundary of ES represented by a single white arrow). Double white arrows point to vacuoles. Black arrows point to nuclei. The female parent of the BC1 triploid was TS50, a colchicine-doubled amphiploid of *T. laxum* (CIMMYT 75-911) x *T. pilosum* (CIMMYT 39-1830) (Leblanc et al 1995). Both *T. laxum* and *T. pilosum* are sexual diploids. The male parent of the BC1 triploid was a different accession of sexual diploid *T. laxum* (CIMMYT 76-916).

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The methods of the 2nd pending patent were applied to *Tripsacum* to convert a genetically-less-stable facultatively-apomictic tetraploid (TS50, a colchicine-doubled amphiploid of *T. laxum* (CIMMYT 75-911) x *T. pilosum* (CIMMYT 39-1830), Leblanc et al 1995) to a near-obligate apomictic triploid with greater genetic stability: 1) genetic stability in the facultatively-apomictic tetraploid was assessed (two sexually-derived progeny plants of the constitution *laxum/pilosum//bravum* showed higher frequency sexual embryo sac formation than the mother apomict), 2) the facultatively-apomictic tetraploid was backcrossed to *T. laxum* to produce a sexually-sterile but apomictically-fertile triploid, and 3) frequency apomictic embryo sac formation was determined in the triploid and was found to be higher than in the tetraploid mother plant. Since triploids are generally sterile, the frequency in which unique but heterozygous combinations of alleles responsible for apomixis recombine and segregate will be much lower. Hence, the triploid plant will exhibit greater genetic stability (see figure).